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(56) Documents Cited
WO 94/08032 A US 5817465 A US 5641864 A

(58) Field of Search
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ONLINE: WPI, EPODOC, JAPIO, CAPLUS, EMBASE, SCISEARCH, MEDLINE, BIOSIS

(54) Abstract Title
Reverse transcriptase formulations

(57) This invention provides a formulation of reverse transcriptase enzymes comprising a reverse transcriptase derived from Avian Myeloblastosis Virus and combined with a reverse transcriptase derived from Moloney Murine Leukaemia Virus, and further provides a method of amplifying nucleic acid sequences by reverse transcription or coupled reverse transcription and polymerase chain reaction.

The formulation enhances sensitivity of RNA detection and increases product yield compared with either of the enzymes used separately.

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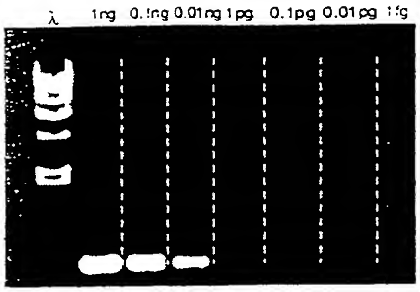


FIGURE 1A

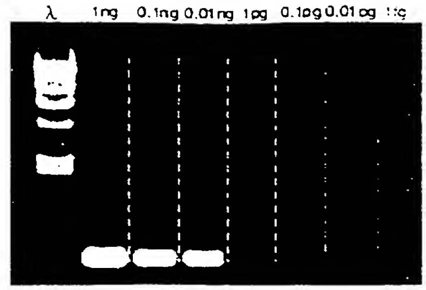


FIGURE 1B

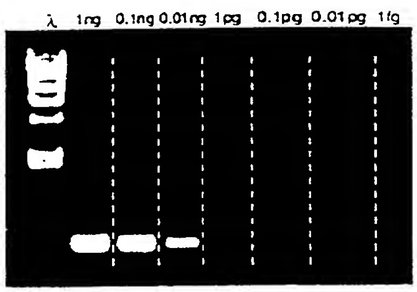


FIGURE 1C

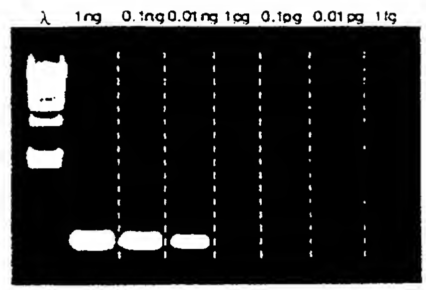


FIGURE 1D

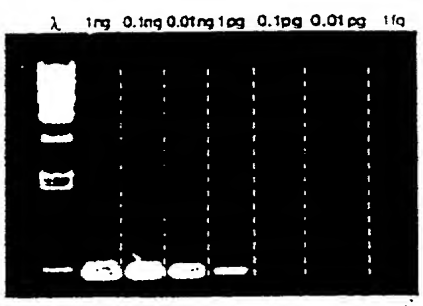
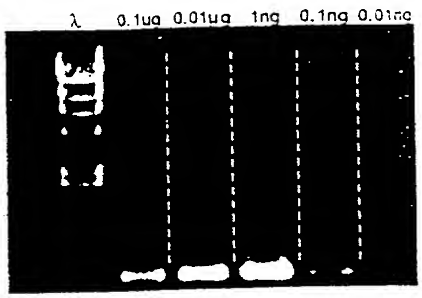
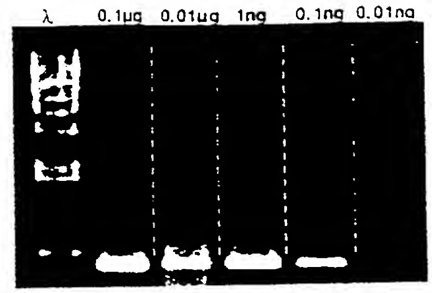


FIGURE 1E



a

FIGURE 2A



b

FIGURE 2B

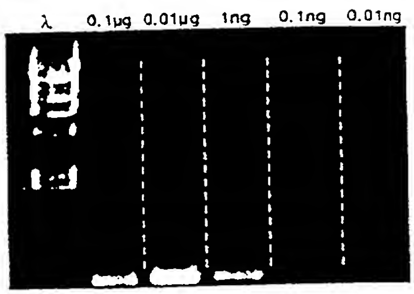


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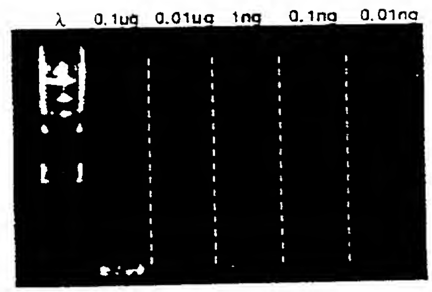
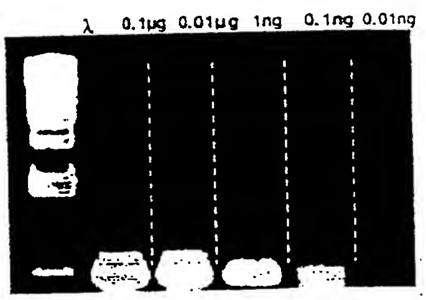
FIGURE 2C



d

FIGURE 2D



e

FIGURE 2E

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IMPROVED REVERSE TRANSCRIPTASE FORMULATIONS

Field of the Invention

The present invention concerns improvements in and relating to reverse transcriptase formulations and includes a novel reverse transcriptase formulation and kit and uses for performing reverse transcription reactions.

Background to the Invention

Gene expression is a fundamental cellular process, in which DNA is transcribed to RNA, prior to protein translation. Gene expression differs from cell to cell, depending on many factors including the environment or state of differentiation of the cell, either with regard to the genes that are expressed, or the levels to which they are expressed. Consequently, measurement of the RNA status of a cell is key to understanding the normal physiological function of that cell, its interaction with other cells and its role in disease processes. Thus, gene expression is routinely analysed by laboratories working in a broad range of research areas.

A technique commonly used for the measurement of RNA in cells or tissues is reverse transcription (RT) and subsequent polymerase chain reaction (PCR) either in one reaction or using separate vessels for the RT and PCR stages. In this process, single-stranded RNA is reverse-transcribed to single-stranded cDNA by the reverse transcriptase (first strand synthesis). A second, complementary strand of DNA is then synthesised/replicated by a thermostable DNA polymerase, creating doubled-stranded cDNA (second strand synthesis). The double-stranded cDNA is subsequently amplified by PCR, whereby specific primer pairs complementary to the cDNA are extended by the DNA polymerase. The amplified DNA product can then be used for analysis and quantification.

Typically, RT and coupled RT-PCR are performed in suitable aqueous buffers that enable the reverse transcriptase and DNA polymerase to function. These buffered systems commonly comprise monovalent cations, such as sodium, potassium or ammonium; and/or magnesium ions, present in the concentration range 1 - 6mM.

One particularly preferred biochemical buffer is Tris (hydroxymethyl) aminomethane hydrochloride, otherwise known as Tris hydrochloride, and which has a useful pH buffering range of between pH 7 and 9.

In addition to buffering agents the typical reverse transcription reaction mixture comprises: (a) deoxy nucleotide triphosphates as the building blocks to form the cDNA;

(b) the RNA template to be transcribed (either total RNA or mRNA) suitably present in the range 10fg - 1μg; and (c) the reverse transcriptase enzyme.

For replication of the cDNA, (d) DNA polymerase and also (e) oligonucleotide primers complementary to the 'sense' (or 'forward') strand or to the 'antisense' (or 'reverse') strand are further provided.

Such a reaction mixture is suitably incubated at 37-60°C for 5-60 minutes, to enable first strand synthesis to be catalysed by the reverse transcriptase. An aliquot of this mixture may then be removed and added to a separate PCR reaction. Whether a two stage separate vessel method is followed, or a coupled RT-PCR method, the reaction proceeds to the PCR stage, where DNA amplification is catalysed by the DNA polymerase.

PCR typically entails an initial incubation at 94 - 95°C for 2 minutes to inactivate the reverse transcriptase and to denature the nucleotide strands. This is followed by 25-35 reaction cycles typically of:

denaturation	94 - 95°C	10 seconds to 2 minutes
annealing	50 - 65°C	10 seconds to 1 minute
extension	68 - 72°C	10 seconds to 10 minutes

The reaction is completed by a final extension step at 68-72°C for 2-10 minutes.

The success of the RT first strand synthesis step is crucial to the overall success of RT-PCR, since the levels of double-stranded DNA produced by PCR are dependent on levels of single-stranded DNA starting material, produced by the reverse transcriptase. As such, any improvement in the efficiency and efficacy of the RT step will ultimately improve the whole technique.

Traditionally, only one reverse transcriptase, for example either AMV or MMuLV, has been used to catalyse first strand synthesis in the RT-PCR reaction. Each is characterised by distinct RNAse H activities and temperature and pH optima. Principally, AMV possesses higher levels of RNAse H activity relative to MMuLV, which is responsible for the degradation of the RNA in the RNA:DNA hybrids. Also, MMuLV has a lower optimal reaction temperature (37 – 42°C) than AMV (48°C - 55°C) which may favour the formation of secondary structure in the RNA. The pH criteria for maximal activity of AMV and MMuLV are also quite different; AMV having an optimal catalytic pH of 8.3, whilst that of MMuLV is 7.6.

A consideration of the temperature and pH optima of the two enzymes would not suggest that a combination of the two would produce enhanced reverse transcriptase activity.

Summary of the Invention

According to the present invention there is provided a formulation having reverse transcriptase activity and comprising a combination of a reverse transcriptase derived from Moloney Murine Leukaemia Virus and a reverse transcriptase derived from Avian Myeloblastosis Virus.

This formulation is suitably adapted to be used in a reverse transcription and subsequent polymerase chain reaction and suitably can be used in a coupled reverse transcription and polymerase chain reaction performed in a single reaction vessel.

Preferably the formulation is packaged together with other reverse transcription reagents in a kit format. Such a kit is preferably adapted to enable the reactions to be performed in a single reaction vessel. The kit format formulation for use in a reverse transcription and polymerase chain reaction suitably has most or all of the reaction components for reverse transcription and polymerase chain reaction supplied in the kit.

Preferably the formulation includes a pH buffering agent to buffer the pH of the formulation, and particularly preferably the buffering agent buffers the pH of the formulation to a pH of 8 or greater and preferably about 8.8. This is notably higher than the normal pH optimum for MMuLV and, at pH 8.8 is even higher than the normal pH optimum for AMV.

Further in contrast to the normal reaction conditions for AMV or MMuLV, the formulation of the present invention operates best at temperatures of the order of 47°C being significantly above the MMuLV optimal range.

The ratio of units of enzyme of Moloney Murine Leukaemia Virus to Myeloblastosis Virus is balanced toward Moloney Murine Leukaemia Virus, preferably being between 3:2 and 7:1 and preferably about 4:1.

The present invention also provides for the use of a formulation as defined above in a reverse transcription reaction and which is suitably further in combination with a polymerase chain reaction.

The use preferably entails the aforementioned reaction conditions of pH greater than 8 and temperature above 42°C and peferably of the order of 47°C.

The invention further provides a kit for use in a reverse transcription reaction and comprising a reverse transcriptase derived from Moloney Murine Leukaemia Virus and a reverse transcriptase derived from Avian Myeloblastosis Virus together with instructions to combine the reverse transcriptases to form a formulation as defined above.

An unexpected and valuable consequence of this novel combined transcriptase formulation, as applied to RT or RT-PCR, is a very significant improvement in sensitivity and product yield over either of the enzymes used separately, for standardised conditions.

Brief Descriptions of the Drawings

Preferred embodiments of the present invention will now be described by way of experimental examples with reference to the accompanying figures in which:

Figures 1A to 1E comprise photographs of an agarose gel electrophoresis result from RT-PCR for each of five different reaction mixtures (a) to (e) in accordance with a first experimental example; and

Figures 2A to 2E comprise photographs of an agarose gel electrophoresis result from RT-PCR for each of five further different reaction mixtures (a) to (e) in accordance with a second experimental example.

Description of Preferred Embodiments

Example 1.

Coupled RT-PCR was performed to illustrate the increased sensitivity of the reverse transcriptase blend, compared to either AMV or MMuLV used separately.

MS2 RNA (Boehringer Mannheim) was serially diluted in 10-fold steps from 1ng to 1fg. Each of these were added to a separate respective reaction mixture containing, in a 50μl volume:

1.25 Units of Taq DNA polymerase

75mM Tris HCl buffer (pH 8.8)

20mM $NH_4(SO_4)$

0.02% (v/v) Tween 20 solvating agent

0.2mM each of dATP, dCTP, dGTP and dTTP deoxynucleotides

10pmol of MS2 primer 1 (5' CTAACGGGGTCGCTGAAT 3')

10pmol of MS2 primer 2 (5' AGGTCGGATGCTTTGTGA 3')

and **either** (a) 0.1 Units AMV, (b) 0.5 Units AMV, (c) 0.4 Units MMuLV, (d) 0.5 Units MMuLV **or** (e) 0.1 + 0.4 Units of the AMV + MMuLV blend reverse transcriptases.

The reaction mixtures were then subjected to RT-PCR under the following conditions:

47°C for 30 minutes

94°C for 2 minutes

40 cycles of 94°C for 20 seconds

60°C for 30 seconds

72°C for 40 seconds

72°C for 5 minutes.

The products were separated by TAE agarose gel electrophoresis on a 1% (w/v) agarose gel alongside a λ Hind III marker for comparison (sizes 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 base pairs. The gel was stained with ethidium bromide, and DNA products visualised using a UV transiluminator (Figure 1). RT-PCR using the MS2 template and primers generates a product of 503bp as can be seen for each of the reaction mixtures (a) to (e) (Figures 1A-E). Additionally, it should be noted that MS2 product is generated from template levels as low as 1pg for each of the reverse transcriptases separately, at either concentration used

(Figure 1A-D). However, a much greater sensitivity was achieved with the blend of reverse transcriptases, detectable product being amplified from as little as 10fg of RNA template (Figure 1E).

Example 2.

Coupled RT-PCR was again performed to illustrate the increased product yield achieved using the reverse transcriptase blend, compared to either AMV or MMuLV used separately but this time using testicular total RNA. The testicular total RNA (Clontech) was serially diluted in 10-fold steps from 0.1μg to 10pg. Each dilution was added to a reaction mixture containing, in a 50μl volume:

1.25 Units of Taq DNA polymerase

75mM Tris HCl (pH 8.8)

20mM $NH_4(SO_4)$

0.02% (v/v) Tween 20

0.2mM each of dATP, dCTP, dGTP and dTTP

25pmol β-Actin Primer 1 (5' AGAGATGGCCACGGCTGCTT 3')

25pmol β-Actin Primer 2 (5' ATTTGCGGTGGACGATGGAG 3')

and **either** (a) 0.1 Units AMV, (b) 0.5 Units AMV, (c) 0.4 Units MMuLV, (d) 0.5 Units MMuLV **or** (e) 0.1 + 0.4 Units of the AMV + MMuLV blend.

The reaction mixtures were then subjected to RT-PCR under the following conditions:

47°C for 30 minutes

94°C for 2 minutes

40 cycles of 94°C for 20 seconds

60°C for 30 seconds

72°C for 40 seconds

72°C for 5 minutes.

The products were separated by TAE agarose gel electrophoresis on a 1% (w/v) agarose gel, the gel stained with ethidium bromide, and DNA products visualised using a UV transiluminator (Figure 2). RT-PCR using β-actin primers and total RNA generated a DNA product of 426bp as can be seen for each of the reaction mixtures (a) to (e) as shown in Figures 2 (A-E).

Product was detectable across the range of RNA template concentrations used (0.1μg to 10pg), with 0.5U of AMV (Figure 2B) and with the blend of AMV + MMuLV (Figure 2E). However the product yield across this RNA template range was much greater for the blend of reverse transcriptases, than for either of the reverse transcriptases used separately, at either concentration.

From the above it is readily apparent that the combination of AMV and MMuLV provides greater sensitivity and product yield when present in the same overall amounts, than either of the enzymes used alone.

CLAIMS

1. A formulation having reverse transcriptase activity and comprising a combination of a reverse transcriptase derived from Moloney Murine Leukaemia Virus and a reverse transcriptase derived from Avian Myeloblastosis Virus.

2. A formulation as in claim 1, that can be used in a reverse transcription and subsequent polymerase chain reaction.

3. A formulation as in claim 2, that can be used in a coupled reverse transcription and polymerase chain reaction performed in a single reaction vessel.

4. A formulation as in claim 1, 2 or 3, wherein the formulation is packaged together with other reverse transcription reagents in a kit format.

5. A formulation as in claim 4 wherein the kit is adapted to enable the reactions to be performed in a single reaction vessel.

6. A formulation in claim 4, that can be used in a reverse transcription and polymerase chain reaction wherein most or all of the reaction components for reverse transcription and polymerase chain reaction are supplied in the kit.

7. A formulation as claimed in any preceding claim wherein the formulation includes a pH buffering agent to buffer the pH of the formulation.

8. A formulation as claimed in claim 7 wherein the buffering agent buffers the pH of the formulation to a pH of 8 or greater and preferably about 8.8.

9. A formulation as claimed in any preceding claim wherein the ratio of units of enzyme of Moloney Murine Leukaemia Virus to Myeloblastosis Virus is balanced toward Moloney Murine Leukaemia Virus, preferably being between 3:2 and 7:1 and preferably about 4:1.

10. Use of a formulation as claimed in any preceding claim in a reverse transcription reaction.

11. Use as claimed in claim 10 further in combination with a polymerase chain reaction.

12. Use as claimed in claim 10 or 11, wherein the pH of the reaction mixture is greater than 8 and/or the temperature of the reaction mixture is greater than 42°C and preferably about 47°C.

13. A kit for use in a reverse transcription reaction and comprising a reverse transcriptase derived from Moloney Murine Leukaemia Virus and a reverse transcriptase derived from Avian Myeloblastosis Virus together with instructions to combine the reverse transcriptases to form a formulation as claimed in any of claims 1 to 9.

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Application No:	GB 0011128.6	**Examiner:**	Dr Rowena Johnson
Claims searched:	All	**Date of search:**	22 September 2000

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.R): G1B (BAC); C3H (HC1)

Int Cl (Ed.7): C12Q 1/68; C12N 9/12

Other: ONLINE: WPI, EPODOC, JAPIO, CAPLUS, EMBASE, SCISEARCH, MEDLINE, BIOSIS

Documents considered to be relevant:

Category	Identity of document and relevant passage		Relevant to claims
A	WO94/08032A	(CEDARS-SINAI MEDICAL CENTRE) See especially page 6 line 2-17 and examples	
A	US5817465A	(MALLET) See especially column 3 line 61-column 4 line 23 and examples	
A	US5641864A	(GELFAND) See especially column 5 line 19-33 and examples	

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
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